ABSTRACT

1. Background and significance of the study

Tuberculosis is amongst the ten leading infectious causes of global mortality, responsible for approximately two billion new cases and 2-3 million deaths annually (Murray et al., 1997; Dye et al., 1999; World Health Organization, 2000). India accounts for 30% of the global burden, with an incidence of 168/1,00,000 and a prevalence rate of 4 per 1,000 (Chakraborty, 1998; World Health Organization, 2004).

The focus of this thesis is on the diagnosis of tuberculosis. The WHO DOTS strategy implemented in 183 countries worldwide focuses on the passive case finding of infectious cases by sputum smear microscopy (World Health Organization, 1998). Microscopy is rapid, cost-effective, targets the most infectious cases, and is highly specific in most high-prevalence settings (Murray et al., 1990). However it is limited by its sensitivity, and sputum smears with less than 5,000 - 10,000 bacilli/ml are negative by sputum smear microscopy (Tomam, 2004).

Culture is sensitive detects upto 100 bacilli/ml, provides a definitive diagnosis, and increases case detection by 30-50% (World Health Organization, 1998). The mean incubation time of 4-8 weeks however, limits its use as a first line diagnostic (Jensen, 1995). Diagnostic algorithms using a combination of indirect diagnostics like response to broad spectrum antibiotics
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and radiological examination are thus used for the diagnosis of smear negative tuberculosis (World Health Organization, 1997).

Symptomatic response to antibiotics does not always exclude tuberculosis, as up to 50% of smear-negative tuberculosis patients have been reported to respond to broad spectrum antibiotics (O'Brien et al., 2003). Chest X-ray is non-specific, fails to diagnose 10%–15% of culture-positive patients, and falsely diagnoses nearly 40% of patients likely not to have active tuberculosis (Tomam, 2004). Their interpretations have limited specificity and inter reader repeatability (Jones et al., 1997).

The implications in disease control are that there is a delay in treatment initiation of patients, increased patient drop out due to repeated visits (Squire et al., 2005) and transmission of disease due to undiagnosed smear negative patients (Behr et al., 1999). Studies carried out in India have shown that a patient of tuberculosis incurs an average total loss of Rs.3,469 and loses 83 work days for diagnosis and treatment (Dholakia, 1996; Rajeshwari et al., 1999).

Conventional diagnostics for tuberculosis have known limitations and their performance is suboptimal especially in the context of the HIV epidemic (Foulds and Brien, 1998; Perkins, 2000). This is reflected in the fact that worldwide the estimated number of sputum smear positive cases have remained constant between 40–50% inspite of increasing DOTS coverage (Dye et al., 2003). WHO initiated the Tuberculosis Diagnostic Initiative in 1997 and is now working in collaboration with the Foundation for Innovative Diagnostics (FIND), for the development, evaluation and demonstration of new diagnostic methods.
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(Word Health Organization, 1997; Foundation for Innovative Diagnostics; www.finddiagnostics.org). Enhanced diagnostic techniques are projected to have a substantial impact on morbidity and mortality in HIV endemic regions reducing tuberculosis prevalence and mortality by 20% (Dowdy et al., 2006). They thus merit further consideration as tuberculosis control strategies.

Various new diagnostics have been described in recent times, some of which show great promise (Pai et al., 2006). Nucleic acid amplification is one of the most extensively studied amongst the newer diagnostics. Various studies using in-house assays or commercial tests (Schirm et al., 1995; Cohen et al., 1998; Aslanzadeh et al., 1998; Kaul, 2001; Piersimonil et al., 2003; Greco et al., 2006; Ozkutuk et al., 2006) including Indian studies (Verma et al., 1994; Dar et al., 1998, Prasad et al., 2001; Tiwari et al., 2003; Chakravorty et al., 2006) have been described to evaluate NAA tests. Sensitivities for smear positive specimens range from 77% to more than 95% and specificities are > 95% (Kaul 2001; Sarmiento et al., 2003; Soini et al., 2001; Woods 2001). Sensitivity for smear-negative patients has been reported to be below 90% (Sarmiento et al., 2003).

Polymerase Chain Reaction (PCR) is a widely used NAA test characterized by its speed, selectivity and sensitivity (Mullis et al., 1987). PCR increases diagnostic certainty, is more patient friendly as it is rapid, and requires lesser patient visits. In addition it is not affected by HIV status of patient. PCR however has its limitations. False-positive PCR results range at rates from 0.8% to 30% and test performance in situations of routine clinical use are inferior to that in controlled study conditions (Rattan, 2000). Various evaluation and multicentre
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Studies stress a great need for standardization (Noordhoek et al., 1996, Noordhoek et al., 2000).

Though several commercial NAA tests are licensed for routine testing of sputum there is uncertainty regarding how these tests should be utilized (Schulger, 2001; Barnes, 1997). Utility and cost effectiveness of such a test in a developing country like India with limited resources in terms of man power with adequate technical expertise and technical infrastructure requires to be studied extremely carefully.

Majority of studies have been performed in developed countries where the burden of tuberculosis is less using primers specific for insertion sequence IS6110. However studies in India have reported that 43% of strains in South India showed no/single copy of this sequence, versus 8-15 copies found in developed countries (Das et al., 1995). With this backdrop, the aim and objectives of this thesis were as follows.

2. Aim and Objectives

The aim of the study is to determine the comparative efficiency and cost effectiveness of PCR to other routine diagnostics for detection of *M. tuberculosis*.

The objectives of the study are:

2.1. To determine the efficiency of routine diagnostics like microscopy and radiological examination used under the Revised National Tuberculosis Control
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Programme (RNTCP) for diagnosis of pulmonary tuberculosis in Pune Municipal Corporation (PMC) area.

2.2. To determine the comparative efficiency of PCR (in terms of sensitivity, specificity, positive and negative predictive value) using MPB64 primers versus other routine diagnostics in the detection of *M. tuberculosis*.

2.3. To determine the cost effectiveness of the PCR to other routine diagnostics like sputum smear microscopy, culture and radiological examination by X-ray.

The study first describes a retrospective analysis of four year data of tuberculosis cases registered under the Revised National Tuberculosis Control Programme (RNTCP) in the Pune Municipal Corporation (PMC) area. This gives a background of the profile of tuberculosis in the PMC area. The study then describes the comparative efficiency and cost effectiveness of a simple low cost, in-house PCR based test using MPB64 primers to other routine diagnostics in this background.

3. Materials and methods

3.1. Materials

3.1.1. Data sources and variables

Data was collected from the RNTCP registers and quarterly reports of the PMC for a four year period, between July 1997 to June 2001. Data
on the total number of chest symptomatics, extrapulmonary and pulmonary tuberculosis cases diagnosed on the basis of smear microscopy (smear positive cases) and cases identified on the basis of radiological examination and other clinical criteria (smear negative cases) was collected.

3.2. Methods

3.2.1. Compilation of data and analysis

Retrospective analysis of total number of chest symptomatics, smear positive and smear negative pulmonary tuberculosis cases registered under the Revised National Tuberculosis Control program (RNTCP) for a four year period, between July 1997 to June 2001 was done using Microsoft EXCEL (version 2000).

Diagnostic and screening efficiency of current routinely used diagnostics was determined. Screening efficiency was defined as the number of pulmonary tuberculosis cases diagnosed using a given diagnostic from amongst all chest symptomatics and diagnostic efficiency was defined as the number of tuberculosis cases diagnosed on the basis of positive smear results or other diagnostics in the diagnostic paradigm amongst all cases.

3.2.2. Collection of samples

A total of 144 samples from as many individuals were tested. Of these, 123 samples were from individuals suspected of having pulmonary tuberculosis, and 21 samples were from hospital patients having a disease other
than tuberculosis. Of the 123 samples, 97 respiratory samples were taken from 97 highly probable tuberculosis suspects who were referred to or who presented at a major Tuberculosis Unit in the PMC area. Samples were examined by routine smear microscopy and the remainder of the same sample was used for culture and PCR. Diagnostic and treatment decisions were made by site physicians according to the Revised National Tuberculosis Control Program (RNTCP) guidelines.

Twenty six respiratory samples (sputum/saliva) were collected from 26 chest symptomatics identified during a morbidity survey carried out in a slum. These individuals reported a productive cough with or without sputum for over three weeks along with one or more cardinal signs of tuberculosis like low grade fever in the evening, weight loss and chest pain. Samples from these individuals were collected by holding health camps (twelve samples from twelve individuals), by referring symptomatics to a nearby municipal clinic (one sample from one individual) or through the collection of samples by health workers (thirteen samples from thirteen individuals).

In addition, 21 samples were taken from 21 hospital patients with a disease other than tuberculosis and examined by all three diagnostics described above. These data were compared with available clinical (n=144) and radiological (n=61) data.

The study was conducted blind. Technicians performing smear microscopy and radiologist/clinician reading X-ray films were blind to results of PCR. All samples were coded and taken to the laboratory for culture and PCR. Culture was performed at the laboratory by the researcher, and the culture bottles
were then transported to the Tuberculosis Unit and interpreted by pathologists at the Unit to avoid bias.

3.2.3. Sample processing, culture and PCR

All samples were processed in a class II biosafety cabinet, using universal biosafety precautions. Samples were processed using either Petroffs's method or N-acetyl-L-cysteine-sodium hydroxide method (Kent et al., 1985). A small amount of the processed pellet was used for culture on Löwenstein Jensen (LJ) medium. DNA was isolated using appropriate methods (Dar et al., 1998). PCR was carried out using MPB64 primers.

Culture results were monitored at one, two and four weeks and reported positive if growth was found after five to six weeks. Positive cultures were confirmed by microscopy for AFB. Cultures were declared negative if there was no growth by twelve weeks. Characterization of Mycobacteria was done at the Corporation laboratory by primary differential tests for atypical Mycobacteria.

X-rays were interpreted by experienced clinicians at the Tuberculosis Unit. Clinical data of symptomatics was collected from the records maintained at the Tuberculosis Units. All symptomatics who were positive by PCR only were followed up by health worker and medical doctor within a year of first presenting to the clinic and medical evaluation was done.

3.2.4. Statistical analysis

Efficiency of microscopy, culture and PCR in terms of sensitivity, specificity, positive and negative predictive valve was done using the gold standards of culture for the culture positive samples and smear microscopy,
combined microbiological data, chest radiographic findings and clinical follow up data for culture negative samples.

3.2.5. Cost analysis

Cost comparison of PCR to sputum smear microscopy, radiological examination by X-ray, and culture was done adopting methods reported by Roos et al., 1998 and Cook et al., 2003. Cost analysis of conventional testing i.e. cost of equipment, chemicals and reagents along with quantities required, and salaries were based on details from the RNTCP. PCR and culture costs were compiled based on actual expenditure incurred in the research laboratory.

The unit costs included material costs, cost of labour and equipment. Costs for each technique were calculated per sample/examination. Material costs were based on the cost and quantity per media/reagent used for performing each test. Equipment costs were obtained from the programme and manufacturers and costed per sample using formula (cost of equipment x rate of depreciation) per total number of samples per year.

Labour cost was calculated on the basis of the yearly salary of the technician. The labour cost was defined as cost per hour with the assumption that the technician worked for eight hours per week for 269 days (excluding 52 Sundays, 24 working holidays and 20 days paid leave). The number of labour minutes required for the performance of each test was multiplied by the derived cost per minute of technologist time to determine the exact labour cost. To
estimate time required test performance was divided into preanalytical, analytical and post analytical activities.

3.2.6. Cost effective analysis

The cost effective analysis compared the routine diagnostic procedure currently in use under the Revised National Tuberculosis Control Programme (Central Tuberculosis Division, 2005) with a theoretical anticipated PCR procedure. The routine diagnostic algorithm followed under the RNTCP is as follows: A tuberculosis suspect is requested to give a spot sputum sample. The next day the patient is asked to return with an early morning sample and an additional sample. Specimens are then sent to the laboratory and the results are sent back. If positive, the patient is registered and put on anti tuberculosis treatment (ATT). If negative then broad spectrum antibiotics are prescribed and if the patient does not respond to these then the patient is asked to repeat three sputum examinations. If positive ATT is started, if negative an X-ray done. If the X-ray is suggestive of tuberculosis then the patient is registered as a smear negative case of tuberculosis and started on ATT. The anticipated PCR procedure assumes that when a person with tuberculosis symptoms presents at the clinic a single sample is collected on the spot. The sample is then subjected to PCR examination.

It was assumed that 100% of smear negative suspects were put on broad spectrum antibiotics and were examined by three repeat sputum examinations, 10% were identified by repeat sputum examination and the remaining were subjected to radiological examination by X-ray. Number of false
positives were assumed to be the same by both procedures i.e. 10%. The cost effectiveness was calculated as (cost of diagnosis of all cases) + cost of treating false positives / correctly diagnosed cases.

The baseline probabilities used were based on previous studies done in the laboratory. Costs related to treatment of false positives were divided into drug costs. Prices of drugs are based on report by Muniyandi et al., 2006. Fixed costs such as facility, space, water, lighting and heat were considered comparable for each method and were ignored. All costs were calculated in Indian rupees.

4. Results

4.1. Profile of tuberculosis in Pune PMC

The efficiency of diagnostics for pulmonary tuberculosis used under the Revised National Tuberculosis Control Programme (sputum smear microscopy, X-ray, and response to broad spectrum antibiotics) was determined. Retrospective analysis of four year data indicated that on an average 6082 ± 2376 chest symptomatics were screened for tuberculosis. An average of 1237 ± 498 symptomatics were diagnosed as tuberculosis cases from this pool of symptomatics. The average number of smear positive and smear negative cases were 800 ± 358 and 438 ± 152.5 respectively. Trend analysis for the four year period indicated a two fold increase in the number of chest symptomatics. A corresponding three fold increase in the smear positive cases and two fold increase in the smear negative cases respectively was seen over the four year period.
An average $20.4 \pm 5.9\%$ symptomatics were diagnosed as pulmonary tuberculosis cases from the chest symptomatics. Of these an average of $13 \pm 5\%$ cases were diagnosed by microscopy (smear positive cases), whilst the remaining $7 \pm 2\%$ smear negative cases were diagnosed on the basis of broad spectrum antibiotics, radiological examination by X-ray and other clinical parameters.

In the PMC, sputum microscopy was used on an average for the diagnosis of between $59\%$ to $68\%$ of all pulmonary tuberculosis cases. The remaining $32\%$ to $41\%$ bacteriologically negative cases could not be diagnosed on the basis of sputum smear microscopy and had to be diagnosed on the basis of other indirect diagnostic tests and clinical findings. The results thus suggested the need for a more sensitive diagnostic for smear negative cases.

### 4.2. Comparative efficiency of polymerase chain reaction (PCR) and other routine diagnostics

PCR for *M. tuberculosis* using primers specific for the MPB64 gene was standardized to detect a 0.2 kb band specific for the *M. tuberculosis* complex. A total of 144 respiratory samples from 144 suspects were examined by smear microscopy, culture on LJ slants, and PCR using primers specific for MPB64.

PCR was positive for $94\%$ of smear positive samples, $86\%$ of the smear negative samples, and $98\%$ of samples positive by culture. In addition, PCR was positive for 17 samples from 17 individuals not diagnosed to have tuberculosis using routine diagnostics. Thirteen such individuals could either be
traced subsequently on follow up or clinical information could be obtained within a year of first presenting to the clinic which suggested that they were highly probable cases of tuberculosis. PCR, culture examination and smear microscopy were performed on 21 individuals with respiratory diseases other than tuberculosis (controls). All samples tested negative for *M. tuberculosis* by all three diagnostics.

In all 71 cases were confirmed as true cases of tuberculosis by the gold standard. Culture and microscopy correctly diagnosed 68% (48 out of 71) and 51% (36 out of 71) of tuberculosis cases respectively. Radiological examination by X-ray was used to diagnose 51% of cases. PCR not only diagnosed maximum 91.5% of the total cases, but also diagnosed additional cases missed by other routine diagnostics. PCR thus proved to be most efficient as a single diagnostic to identify maximum number of tuberculosis cases with an efficiency of 89% compared to culture and microscopy 83% and 76% respectively. PCR as a single diagnostic identified maximum cases in the shortest possible turnover time of 24-48 hours. Comparison of PCR to conventional methods revealed a significant difference using McNemar's Chi square test ($\chi^2 = 5.26$, df = 1, $P < 0.05$).

PCR was useful in the diagnosis of tuberculosis especially amongst symptomatics who could not expectorate a proper sputum sample, and amongst contacts of tuberculosis cases. 31% of symptomatics could not expectorate a proper sputum sample. PCR was positive for eight such cases versus one by microscopy, four by culture and six by radiological examination. PCR was useful
in diagnosing contacts of tuberculosis cases (three by routine diagnostics versus eight by PCR, of which five were confirmed as true cases by the gold standard).

4.3. Comparative cost analysis of PCR to routine diagnostics

Cost comparison of PCR to sputum smear microscopy, X-ray and culture was done. Costs for each of the diagnostic techniques were calculated per sample/examination. The material costs for sputum smear microscopy, culture, X-ray and PCR were Rs. 6.69, Rs. 14.85, Rs. 35.61 and Rs. 106.27 respectively. Equipment cost per sample/examination for smear microscopy, culture, X-ray and PCR were Rs. 1.97, Rs. 1.87, Rs. 10.60, and Rs. 5.26 respectively. Labour costs for sputum smear microscopy, culture, X-ray and PCR per sample were Rs. 9.43, Rs. 8.60, Rs. 9.36 and Rs. 20.27 respectively.

The total unit cost (equipment costs, material costs and labour costs) per sample were thus Rs. 18, Rs. 25, Rs. 56 and Rs. 132 respectively for microscopy, culture, X-ray and PCR respectively. The cost for a course of broad spectrum antibiotics for a ten day period was Rs. 9. PCR was thus seven times more expensive than single smear examination and two times more expensive than radiological examination by X-ray. The cost per sample for diagnosis of a smear positive case equaled Rs. 54 (three sputum smear examinations) while that of a smear negative equaled Rs. 173 respectively (three sputum smear examinations, a ten day regimen of broad spectrum antibiotics, three repeat sputum examinations and cost of radiological examination by X-ray). Thus ratio of PCR to routine diagnostics for diagnosis of smear positive case of tuberculosis was 2.4 while and that for smear negative case was 0.8.
Cost effective analysis comparing routine diagnostic procedure currently in use under the RNTCP with a theoretical anticipated PCR procedure was done. Retrospective analysis of four year data indicated that on an average 6000 tuberculosis suspects were screened per year, of which 1200 (20%) were diagnosed with tuberculosis. Of these 780 (65%) were smear positive cases on first examination, 120 (10%) were identified on re-examination by smear microscopy while the remaining 25% (300) were smear negative cases.

Sputum smear microscopy could thus be used to diagnose 900 cases after screening 6000 symptomatics. The remaining 5100 symptomatics were screened with broad spectrum antibiotics and X-ray, so that 25% i.e. 300 smear negative cases could be diagnosed. The total costs for screening a batch of 6000 patients using the routine diagnostic procedure was thus Rs. 9,38,460. The false positive rate by both techniques was assumed to be 10% of smear negative i.e. 30 cases. Thus routine diagnostics correctly identified 1,170 cases (smear positives + smear negatives – false positives) as having tuberculosis. The unit cost for treating a smear positive case was taken as Rs. 392 and that of a smear negative case as Rs. 277 (Muniyandi et al., 2006). Cost of treating a case of tuberculosis was considered as an average of the two i.e. Rs. 335. The cost effectiveness of the routine diagnostic procedure (cost of diagnosis of all cases) + drug costs of treating false positives (Rs 335)/ number of tuberculosis cases correctly identified was thus Rs. 811. The total costs involved in screening using PCR were calculated at Rs. 7,92,000 (6000 x 132). Out of these suspects 1,427 were diagnosed as having tuberculosis on the basis of sensitivity data obtained from
PCR standardized in the laboratory, and 143 were falsely diagnosed as having tuberculosis. The cost effectiveness of the PCR procedure was Rs. 654 per correctly diagnosed tuberculosis case. The cost effectiveness ratio using the routine diagnostic procedure versus PCR was 1.2.

5. Summary of key findings

An increasing trend (two fold) was seen in the number of symptomatics screened for tuberculosis in the PMC area over a four year period. The increase in the number of sputum smear positive cases and smear negative cases was similar (2.8 and 2.5 fold respectively), indicating that smear negative cases constituted a significant portion of the tuberculous case load. On an average 20.4 ± 5.9% symptomatics in the PMC area were diagnosed as pulmonary tuberculosis cases, 13 ± 5% by microscopy, the remaining 7 ± 2% were diagnosed on the basis of broad spectrum antibiotics, X-ray and other clinical parameters.

In the RNTCP of PMC, microscopy was used to detect two-thirds of pulmonary tuberculosis cases. However, a progressively increasing load of symptomatics had to be screened, using indirect tests, in order to screen out the remaining one-third of sputum smear negative cases. These observations raised the possibility that as attendance of symptomatics to the centres for diagnosis improved, a comparatively greater proportion of resources are likely to be diverted to screening chest symptomatics in order to identify sputum negative cases of pulmonary tuberculosis. The need for a suitable and sensitive diagnostic was thus obvious from this data.
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PCR proved to be the most sensitive diagnostic with a sensitivity of 91.5% versus microscopy 51% and culture 68%. In addition PCR detected 17 samples negative by other routine diagnostics seven of which were considered as true cases. Three of the remaining six were family contacts of tuberculosis cases, one immunocompromised individual with history of extrapulmonary tuberculosis was deceased, and two were highly probable cases. However due to lack of a suitable gold standard they had to be considered false positives.

PCR was positive for maximum cases amongst those who were not able to expectorate a proper sputum sample. Out of 45 such samples PCR was positive for 12 versus seven by routine diagnostics. Three of the additional seven detected by PCR were considered true positive on follow up analysis. In addition PCR was also useful in diagnosis of tuberculosis amongst contacts. Samples of three contacts were positive by routine diagnostics, while eight were positive by PCR. Thus PCR detected maximum cases in the shortest turnover time (less than 24 hrs) compared to other routine diagnostics.

Comparative analysis of various cost components i.e. the equipment costs, material costs and labour costs per sample for the four techniques indicated that PCR was seven times more expensive than smear microscopy and two times more expensive than radiological examination by X-ray. PCR was 2.4 more expensive than microscopy for diagnosis of smear positive samples. However diagnosis of smear negative cases by routine diagnostics was 1.2 more expensive than PCR. The cost per correctly diagnosed case by routine
diagnostics was Rs. 811 versus Rs. 654 by PCR. Thus the cost effective ratio of routine diagnostics to PCR was 1.2.

6. Conclusions

- PCR using primers for MPB64 gene is a rapid, and sensitive diagnostic for detection of *M. tuberculosis* in clinically suspect symptomatics compared to routine diagnostics. It enhances diagnostic certainty but should be interpreted in a clinical context. Absence of a suitable gold standard remains one of the strong limitations of PCR.

- PCR increases diagnostic certainty for tuberculosis especially amongst individuals who cannot expectorate a proper sputum sample, and amongst tuberculosis contacts.

- PCR has a potential to be a cost effective diagnostic in Indian situations when primers and reagents which are not patented are used and where labour is comparatively cheaper than in developed countries.

- Various models can be proposed as an outcome of this work and integrated in future studies to determine how this diagnostic may be used in the realistic set up of public health programme in a developing country like India.